

The EGF-CFC Protein One-Eyed Pinhead Is Essential for Nodal Signaling

Kira Gritsman,[†] Jiaojiao Zhang,[†]
Simon Cheng, Elizabeth Heckscher,
William S. Talbot, and Alexander F. Schier*
Developmental Genetics Program
Skirball Institute of Biomolecular Medicine
Department of Cell Biology
New York University School of Medicine
New York, New York 10016

Summary

The zebrafish EGF-CFC gene *one-eyed pinhead* (*oep*) is required zygotically for the formation of the ventral neuroectoderm, endoderm, and prechordal plate. Here we report that embryos lacking both maternal and zygotic *Oep* activity are defective in germ layer formation, organizer development, and the positioning of the anterior–posterior axis. An identical phenotype is displayed by double mutants for the *nodal*-related genes *squint* and *cyclops*. Mutations in *oep* eliminate the response to *Squint* and *Cyclops* overexpression but are suppressed by expression of Activin and activated forms of the type I receptor ActRIB and Smad2. Expression of the murine EGF-CFC gene *cripto* rescues *oep* mutants. These results suggest a conserved role for EGF-CFC proteins as essential extracellular cofactors for Nodal signaling during vertebrate development.

Introduction

The vertebrate body plan is established during early embryogenesis when the three germ layers are generated, the embryonic axes are patterned, and distinct cell types are specified. Genetic analyses in mouse and zebrafish have highlighted the fundamental role of signaling by the TGF β molecule Nodal during mesoderm and endoderm development (Zhou et al., 1993; Conlon et al., 1994; Feldman et al., 1998). Mouse *nodal* mutants are impaired in germ layer formation, as evidenced by the absence of the primitive streak and a severe reduction of mesoderm and endoderm (Zhou et al., 1993; Conlon et al., 1994). Zebrafish double mutants for the *nodal*-related genes *squint* (*sqt*) and *cyclops* (*cyc*) also are severely disrupted during gastrulation and lack mesodermal and endodermal derivatives (Feldman et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998).

EGF-CFC proteins have recently been recognized as a novel class of extracellular factors that are essential during zebrafish and mouse development (Ding et al., 1998; Zhang et al., 1998). The EGF-CFC proteins *Oep* (zebrafish), *Cripto* (mouse), *Cryptic* (mouse), and *FRL1* (frog) contain a signal sequence, a characteristic EGF-like domain, a second cysteine-rich region called the

CFC domain, and a hydrophobic C terminus (Kinoshita et al., 1995; Shen et al., 1997; Zhang et al., 1998; reviewed in Salomon et al., 1999). In zebrafish, mutations in *one-eyed pinhead* (*oep*) result in cyclopia and absence of endoderm, prechordal plate, and ventral neuroectoderm (Hammerschmidt et al., 1996; Schier et al., 1996, 1997; Strähle et al., 1997). Mutations in the mouse EGF-CFC gene *cripto* affect the development of mesoderm and visceral (extraembryonic) endoderm and change the orientation of the anterior–posterior axis (Ding et al., 1998).

The signals or receptors that interact with EGF-CFC proteins have remained elusive. In cell culture assays, soluble *Cripto* protein induces responses such as Ras and MAPK activation (reviewed in Salomon et al., 1999), and in a yeast phosphorylation assay, FRL1 can activate the FGF receptor (Kinoshita et al., 1995). As no direct interaction of *Cripto* or FRL1 with EGF or FGF receptors has been detectable, it has been proposed that EGF-CFC proteins might be a novel class of instructive signals acting via unidentified receptors that activate the Ras/Raf/MEK/MAPK pathway (Salomon et al., 1999). However, the phenotypes of *cripto* and *oep* mutants are quite distinct from those induced by blocking Ras signaling (reviewed in Harland and Gerhart, 1997), suggesting that EGF-CFC proteins might not be required for the activation of this pathway in vivo. Furthermore, the autonomous and permissive role of zebrafish *Oep* appears inconsistent with a model wherein all EGF-CFC proteins act as instructive signals (Schier et al., 1997; Strähle et al., 1997; Zhang et al., 1998).

The results reported here provide evidence that EGF-CFC proteins act as essential extracellular cofactors for Nodal signaling during vertebrate development. By generating embryos that lack both maternal and zygotic *Oep* activity, we found that *Oep* is required not only for endoderm, prechordal plate, and ventral neuroectoderm development but also for head and trunk mesoderm formation, organizer development, and positioning of the anterior–posterior axis. Maternal-zygotic *oep* mutants are strikingly similar to double mutants for the *nodal*-related genes *sqt* and *cyc* and are unresponsive to overexpression of Nodal signals. Activation of putative downstream components of Nodal signaling suppresses the phenotype caused by loss of *Oep* activity. Comparison with mouse *cripto* mutants and the finding that *cripto* and *FRL1* mRNA injection can rescue *oep* mutants indicate that EGF-CFC proteins have a general role in Nodal signaling and that conserved molecular mechanisms underlie germ layer formation and the positioning of the anterior–posterior axis in vertebrates.

Results

Severe Patterning Defects in Embryos Lacking Maternal and Zygotic *Oep* Activity

The *oep* gene is expressed both maternally and zygotically (Zhang et al., 1998). To investigate the role of maternal *oep* during embryogenesis, we generated females

* To whom correspondence should be addressed (e-mail: schier@saturn.med.nyu.edu).

[†] These authors contributed equally to this work.

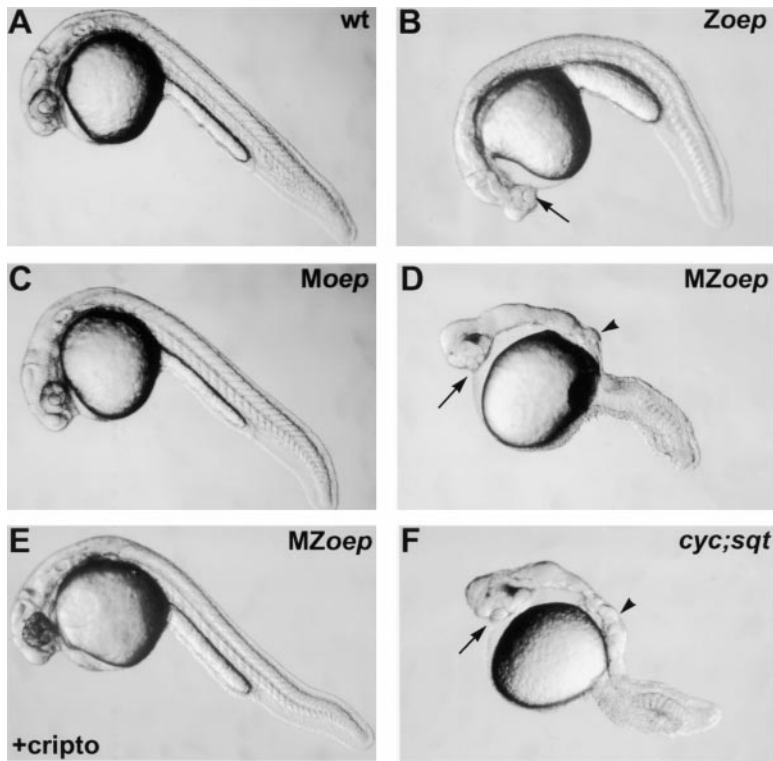


Figure 1. Maternal-Zygotic Effect of *oep*
(A–F) Live embryos at 30 hr postfertilization (hpf), anterior to the left, dorsal up. (A) Wild-type embryo. (B) Zygotic *oep*^{z57} mutant (*Zoe*) with severe cyclopia and ventral forebrain defects (arrow). (C) Maternal *oep*^{z57} mutant (*Moep*). (D) Maternal-zygotic *oep* mutant (*MZoe*). Note the severe defects in mesoderm and endoderm formation and similarity to *sqt;cyc* double mutant embryos (F). Somites form in the tail. The location of ear and cyclopic eye are highlighted by arrowhead and black arrow, respectively. (E) Phenotype of *MZoe* mutants after microinjection of 25 pg mRNA encoding mouse *Cripto* at the 1–4 cell stage. Nineteen out of twenty-two *MZoe* embryos injected with *cripto* mRNA appeared fully rescued at 28 hpf.

homozygous for the null allele *oep*^{z57} using *oep* mRNA-mediated rescue of zygotic *oep* mutants. We found that rescued embryos can develop into fertile homozygous *oep/oep* adults. To generate embryos lacking maternal but not zygotic *Oep* activity, we crossed *oep/oep* females to wild-type males. The resulting embryos (*Moep* mutants) develop normally (Figure 1C), establishing that maternal *oep* is not required in the presence of zygotic *Oep* activity.

The presence of maternal *oep* mRNA overlaps with zygotic *oep* mRNA expression during blastula stages (Zhang et al., 1998) and might partially compensate for the loss of zygotic *oep*, a property of several *Drosophila* patterning genes (Jimenez and Campos-Ortega, 1982; Perrimon et al., 1984). We thus generated embryos lacking both maternal and zygotic *Oep* activity (*MZoe* mutants) by crossing *oep/oep* homozygous females to *oep/oep* homozygous or *oep/+* heterozygous males (Figure 1D). Strikingly, *MZoe* mutant embryos are very similar to double mutants for *sqt* and *cyc*, two zebrafish *nodal*-related genes (Figure 1F). Mutant embryos display severe defects not seen in *Zoe* mutants, including lack of the notochord, pronephros, and blood. A variable number of somites that form in the tail region are the only nonectodermal structures detected in *MZoe* mutants (Figure 1D). Anterior–posterior patterning is apparent in the brain, with a single median eye at the anterior end and an otic vesicle more posteriorly. Injection of *oep* mRNA rescues *MZoe* mutants to adulthood (data not shown), confirming that these defects are caused by the absence of *Oep* activity. These results reveal novel roles for *oep* in embryonic patterning.

oep Is Required for Involution of Marginal Cells and Positioning of the Anterior–Posterior Axis

To study the morphogenesis of *MZoe* mutants, we carried out a time course analysis (Figure 2). The phenotype is clearly apparent at the onset of gastrulation, when the embryonic shield, a thickening at the dorsal margin (Kimmel et al., 1995), does not form in *MZoe* mutants (Figure 2F). In addition, involution of marginal cells, which normally form the mesendodermal hypoblast layer, is not detectable in *MZoe* mutants (Figures 2G and 2H). The movements of epiboly and convergence, which drive cells vegetally and dorsally, respectively, do occur, albeit abnormally. Fewer cells reside at the vegetal pole (Figures 2Q and 3A6), and an abnormal thickening at the dorsal–medial region of the embryo is visible at midgastrulation (Figure 2I). Time course analysis and marker gene expression reveals that this region develops into the brain of *MZoe* mutants (Figures 2J, 2P–2T, 3B2, 3B4, and 3B6). Thus, the anterior-most region in *MZoe* mutants is displaced ~90° vegetally from its wild-type position at the animal pole (compare Figures 2K and 2P and 3B1 and 3B2). Concurrently, the tail in *MZoe* mutants is located at a more animal–ventral position than in wild-type embryos (Figures 2P–2T). Expression of the hindbrain marker *krox20* (Figures 3A6 and 3B6) and the tail marker *ntl/brachyury* (Figure 3C6) at the end of gastrulation indicates that the presumptive hindbrain and tail are located close to each other at the dorsal and ventral vegetal margin, respectively. Subsequent extension movements of the head and tail occur, but the positioning of the anterior–posterior axis of *MZoe* mutants with respect to the animal–vegetal axis remains rotated as compared to wild-type embryos

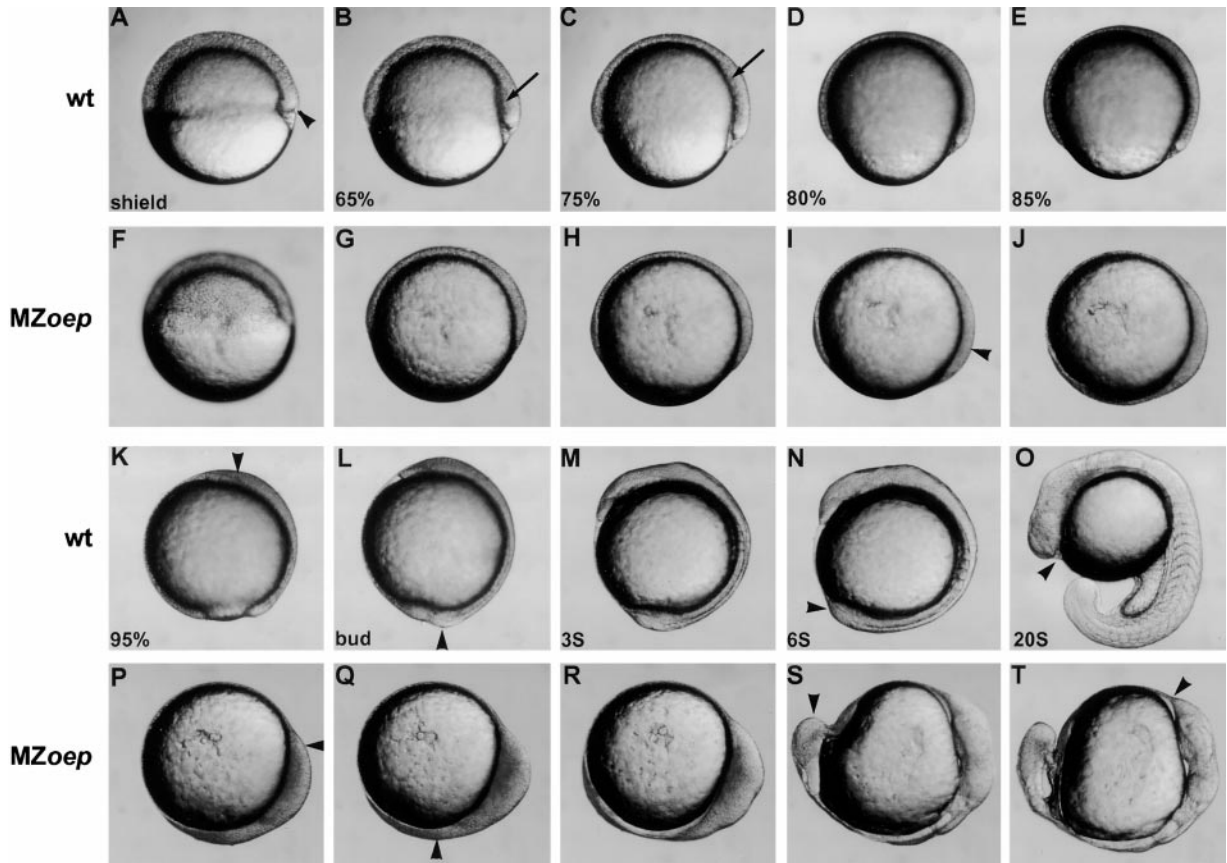


Figure 2. Morphogenesis of Maternal-Zygotic *oep* Mutants

(A–E and K–O) Wild-type embryo; same embryo is shown at all stages. (F–J and P–T) *MZoep* mutant embryo; same embryo is shown at all stages. Animal pole is up and dorsal is to the right. 3S, 6S, and 20S indicate 3-somite, 6-somite, and 20-somite stage, respectively. The embryonic shield, marked by an arrowhead in (A), fails to form in *MZoep* embryos at 50% epiboly (F). Note the formation of the hypoblast layer in wild-type (arrows in [B] and [C]) but not *MZoep* mutant embryos (G and H) and the vegetal–dorsal thickening in *MZoep* mutants (arrowhead in [I]). In wild-type embryos, the involuting hypoblast migrates anteriorly and the head forms at the animal pole (arrowhead in [K]). In *MZoep* mutant embryos, a 90° vegetalward displacement of the head is apparent (arrowhead in [P]). The tailbud forms at the vegetal pole at the end of epiboly in wild-type embryos (arrowhead in [L]). In *MZoep* embryos, a vegetal reduction of cells (arrowhead in [Q]) is apparent at the presumptive head/tail border region. At later stages, the displacement of the head is still evident (compare position of arrowheads in [O] and [T]), while the tail is also displaced anteriorly and ventrally (compare arrowheads in [N] and [S]).

(compare Figures 2O and 2T). These results indicate that *MZoep* mutants acquire dorsal–ventral and anterior–posterior polarity during gastrulation and that germ layer formation and normal positioning of the anterior–posterior axis is severely impaired.

oep Is Essential for the Proper Formation of the Organizer and Mesendoderm

To characterize in more detail the functions of *oep* in the formation of mesoderm and endoderm, we analyzed the expression of marker genes during gastrula stages and somitogenesis. Markers for endoderm precursors (*axial*, Figure 3C2), notochord (*ntl/brachyury*, Figure 3C6; *axial*, Figure 3C2), prechordal plate (*hgg1*, Figure 3C4), paraxial mesoderm (*myoD*, Figure 3D4; α -tropomyosin, Figure 3D6), intermediate mesoderm (*pax2.1*, data not shown), and lateral mesoderm (*gata1*, Figure 3D2) are not expressed in the head and trunk of *MZoep* mutants,

confirming the severe disruption of mesendodermal development.

To study dorsal–ventral and animal–vegetal patterning, we analyzed the expression of ventral markers (*BMP4*, Figure 3F2; *gata2*, Figure 3F4), dorsal markers (*goosecoid* [*gsc*], Figure 3G2; *floating head* [*flh*], Figure 3G4; *chordin*, Figure 3G6; *dharma/bozozok*, Figure 3F6), and marginal markers (*ntl/brachyury*, Figure 3E2; *wnt11*, Figure 3E4; *FGF8*, Figure 3E6) during blastula and gastrula stages. Extending our morphological observations, there is clear dorsal–ventral polarity in *MZoep* mutants. Marginal–most cells do not express *FGF8*, preceding the aberrant germ layer formation in *MZoep* mutants. The most severe defects are detected at the dorsal margin, the location of the presumptive organizer. Expression of both *ntl/brachyury* and *wnt11* is absent in this region, and the expression of the organizer genes *flh* and *gsc* is strongly reduced. Dorsal expression of *chordin* is

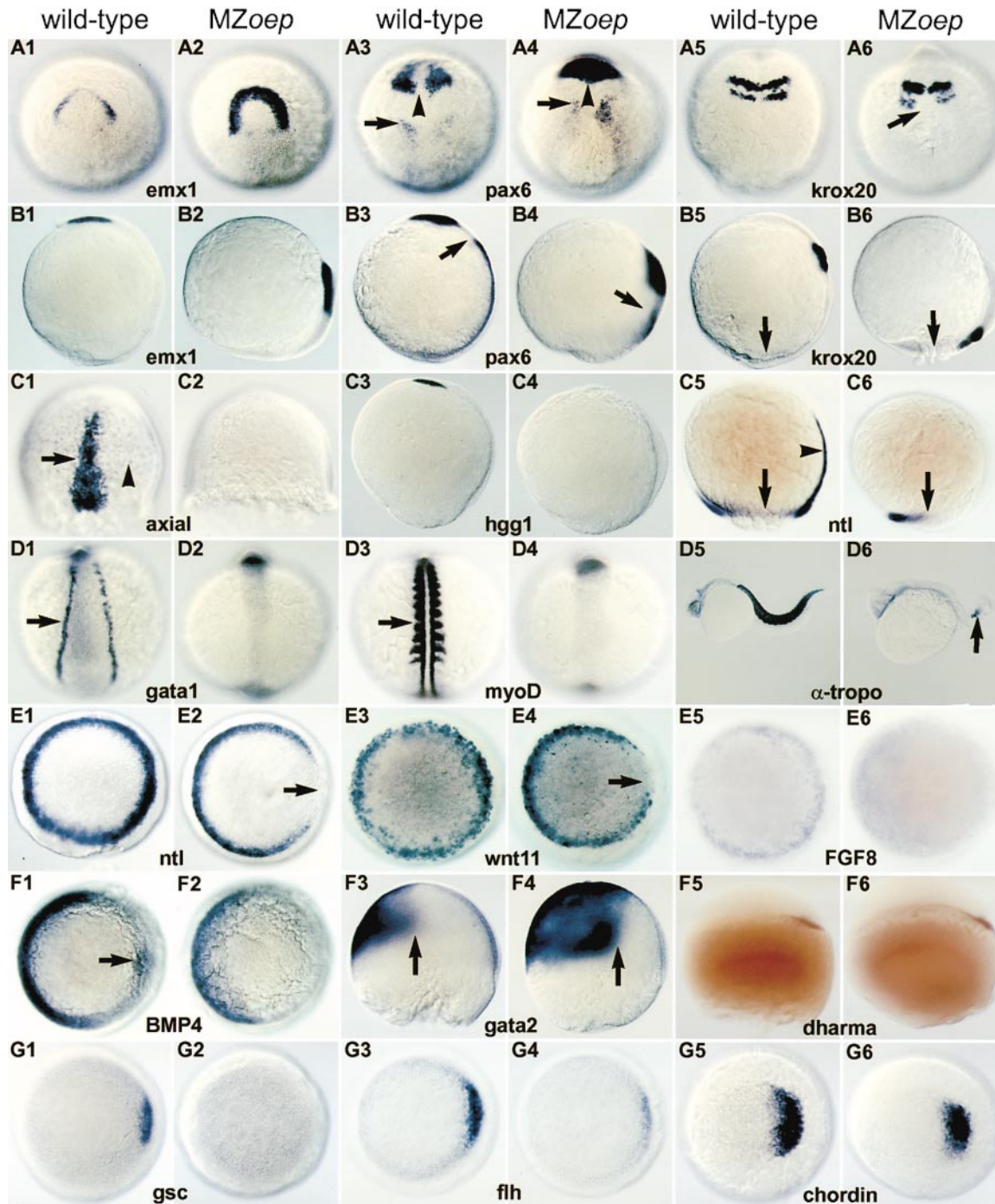


Figure 3. Marker Gene Expression in Maternal-Zygotic *oep* Mutants

Expression of mRNA was detected by whole-mount in situ hybridization. (A1–A6) Dorsal view of head region. (B1–B6) Lateral view with vegetal pole down. (A1, A2, B1, and B2) Expression of *emx1* in anterior forebrain at 2-somite stage (2S). Note expansion in MZ*oep* mutant embryo (A2). (A3, A4, B3, and B4) Expression of *pax6* at 1S in forebrain and presumptive eye fields and hindbrain (arrow). Bilateral *pax6* expression domains are fused in MZ*oep* mutants (arrowhead). (A5, A6, B5, and B6) Expression of *krox20* at 2S in rhombomeres 3 and 5. Note the vegetal gap posterior to rhombomere 4 (arrow in A6). Note the displacement of the expression domains of *emx1*, *pax6*, and *krox20* toward the vegetal pole, with *krox20* located at the dorsal-vegetal side (arrow in [B5] and [B6]). (C1 and C2) Expression of *axial* at 70% epiboly in axial mesoderm (arrow) and endoderm progenitors (arrowhead). (C3 and C4) Expression of *hgg1* in anterior prechordal plate at 90% epiboly. (C5 and C6) Lateral view of *ntl/brachyury* expression in notochord (arrowhead) and margin (arrow) at 90% epiboly. Note expression in MZ*oep* mutants at the lateral and ventral but not dorsal margin (arrow in C6). (D1 and D2) Expression of *gata1* at 9S in blood progenitors (arrow). (D3 and D4) Expression of *myoD* at 9S stage in muscle progenitors (arrow). (D5 and D6) Expression of α -tropomyosin at 24 hpf in muscle. Note expression in tail of MZ*oep* mutants (arrow). (E1 and E2) Expression of *ntl/brachyury* at 45% epiboly. A dorsal gap is found in MZ*oep* mutants (arrow). (E3 and E4) Expression of *wnt11* at 30% epiboly, including dorsal gap in MZ*oep* mutants (arrow). (E5 and E6) Expression of *FGF8* at 30% epiboly. Note the absence of *FGF8* expression in MZ*oep* mutants. (F1 and F2) Expression of *BMP4* at shield stage in ventral region and dorsal-

maintained at reduced levels in MZ*oep* mutants, establishing that some dorsal gene expression can occur in the absence of *oep* activity. Previous studies have shown that the dorsal yolk syncytial layer (YSL), an extra-embryonic syncytium that underlies the developing embryo after the midblastula stage, is involved in inducing overlying dorsal mesoderm (reviewed in Schier and Talbot, 1998). The homeobox gene *dharmabozozok* is expressed in the dorsal YSL and is thought to mediate part of its inducing activity (Koos and Ho, 1998; Yamanaka et al., 1998; Fekany et al., 1999). The *dharmabozozok* gene is expressed in the dorsal extraembryonic region of MZ*oep* mutants, indicating that dorsal induction is initiated in MZ*oep* mutants.

To study the requirement of *oep* in neural patterning, we analyzed the expression of *emx1* (Figures 3A2 and 3B2), *pax6* (Figures 3A4 and 3B4), *otx2* (data not shown), *pax2.1* (data not shown), and *krox20* (Figures 3A6 and 3B6) in the developing neural plate. Anterior–posterior polarity of the brain is apparent, and the expression domain of anterior markers such as *emx1* is expanded. No ventral neuroectoderm forms, as judged from the absence of *shh* expression, but dorsally derived melanocytes develop in MZ*oep* mutant embryos (data not shown). These results indicate that anterior–posterior patterning of the neural plate occurs despite severe defects in germ layer formation.

oep Is Required for Nodal Signaling

The MZ*oep* mutant phenotype is strikingly similar to that of double mutants that lack the *nodal*-related genes *sqt* and *cyc* (Figure 1F; Feldman et al., 1998), implying that Oep and Sqt/Cyc act in a common pathway. Furthermore, the defects in floor plate formation and ventral forebrain development in zygotic *oep* mutants also correlate with proposed functions of Nodal signaling in these processes (Hatta et al., 1991; Rebagliati et al., 1998a; Sampath et al., 1998). The observation that Oep acts cell autonomously (Schier et al., 1997; Strahle et al., 1997), whereas Nodal signals can act nonautonomously (Erter et al., 1998; Feldman et al., 1998; Sampath et al., 1998), suggested that Oep is required for cells to receive Nodal signals. To test this idea, we analyzed the epistatic relationship of *nodal* and *oep* by injecting mRNAs encoding Sqt, Cyc, or mouse Nodal into wild-type and MZ*oep* mutants (Figure 4; Table 1). Misexpression of these factors in wild-type embryos results in severe dorsalization (Figures 4C, 4E, and 4G) and ectopic expression of *gsc* (Figures 4K, 4M, and 4O). In striking contrast, no rescue or dorsalization is observed in MZ*oep* mutants, even at doses that are 1000× higher than those sufficient for dorsalization of wild-type embryos (Figures 4D, 4F, 4H, 4L, 4N, and 4P; Table 1). These results establish that Oep is essential for Nodal signaling during embryogenesis.

Rescue of *oep* Mutants by Activation of the Smad2 Signaling Pathway

To determine what step in the Nodal signaling pathway requires Oep, we analyzed the relationship of Oep with putative downstream components of Nodal signaling. While the targets of Nodal have not been identified definitively, studies in mouse and frog have suggested that Nodal signaling is mediated by a TGFβ signaling pathway, possibly involving the type IB Activin receptor (ActRIB) and the transcription factor Smad2 (Gu et al., 1998; Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998; reviewed in Harland and Gerhart, 1997; Massagué, 1998; Whitman, 1998). To determine if Oep is essential for the response to these factors, we injected activated versions of ActRIB and Smad2 into MZ*oep* mutant embryos and assayed for phenotypic rescue and induction of *gsc* expression (Figure 5; Table 1). Expression of constitutively activated ActRIB (Figures 5E–5H) or Smad2 (Figures 5I–5L) rescues the MZ*oep* mutant phenotypes, including *gsc* expression (Figures 5H and 5L). These results indicate that Oep is essential for Nodal to activate a TGFβ signal transduction pathway.

Previous *in vitro* studies have suggested that the EGF-CFC proteins Cripto and FRL1 might be involved in the Ras/Raf/MEK/MAPK pathway (reviewed in Salomon et al., 1999). However, the maternal-zygotic *oep* phenotype is very distinct from those induced by loss of Ras activity (reviewed in Harland and Gerhart, 1997). To further test if the *oep* mutant phenotype is caused by loss of Ras/Raf/MEK/MAPK signaling, we expressed an activated form of Ras in MZ*oep* mutants (Whitman and Melton, 1992). Both wild-type and MZ*oep* mutant embryos displayed a reduction of anterior structures, and no rescue of the mutant phenotype was observed (Figures 5Q–5T), further supporting a specific requirement for Oep in Nodal but not Ras/Raf/MEK/MAPK signaling.

Rescue of *oep* Mutants by Activin

To further study the specificity of the interaction of Oep with TGFβ signals, we assayed the effects of Activin in MZ*oep* mutants. Activin activates the Activin receptors (ActRIB, ActRII, ActRIIB) and Smad2 and, like Nodal, has been shown to be a mesoderm inducer and dorsalizer (reviewed in Harland and Gerhart, 1997; Massagué, 1998; Whitman, 1998). Surprisingly, injection of *activin* mRNA into MZ*oep* mutant embryos rescues the mutant phenotypes, causing some of the rescued embryos to look almost like wild type (Figures 5M–5P; Table 1). This result indicates that Oep is specifically required for the activity of Nodal but not Activin. In addition, the downstream TGFβ signaling components that can bring about mesendoderm formation and dorsal induction are present and can be activated in MZ*oep* mutants. These results also suggest that the active levels of endogenous

most area (arrow) in wild type and only in ventral region in MZ*oep* mutant. (F3 and F4) Expression of *gata2* at 70% epiboly, including dorsal expansion of expression domain in MZ*oep* mutants (arrow). (F5 and F6) Expression of *dharmabozozok* (Koos and Ho, 1998; Yamanaka et al., 1998) in the yolk syncytial layer at dome stage. (G1 and G2) Expression of *gooseoid* at 30% epiboly. (G3 and G4) Expression of *floating head* at 45% epiboly. (G5 and G6) Expression of *chordin* at sphere stage. *chordin* expression is also maintained in *cyc;sqt* double mutants (B. Feldman, A. F. S., and W. S. T., unpublished results). (A1–A6, C1, C2, and D1–D4) Dorsal view, anterior top. (B1–B6, C3–C6, and F3–F6) Lateral view, dorsal right. (D5 and D6) Lateral view, anterior left. (E1–E4, F1, F2, and G1–G6) Animal pole view, dorsal right. (E5 and E6) Animal pole view.

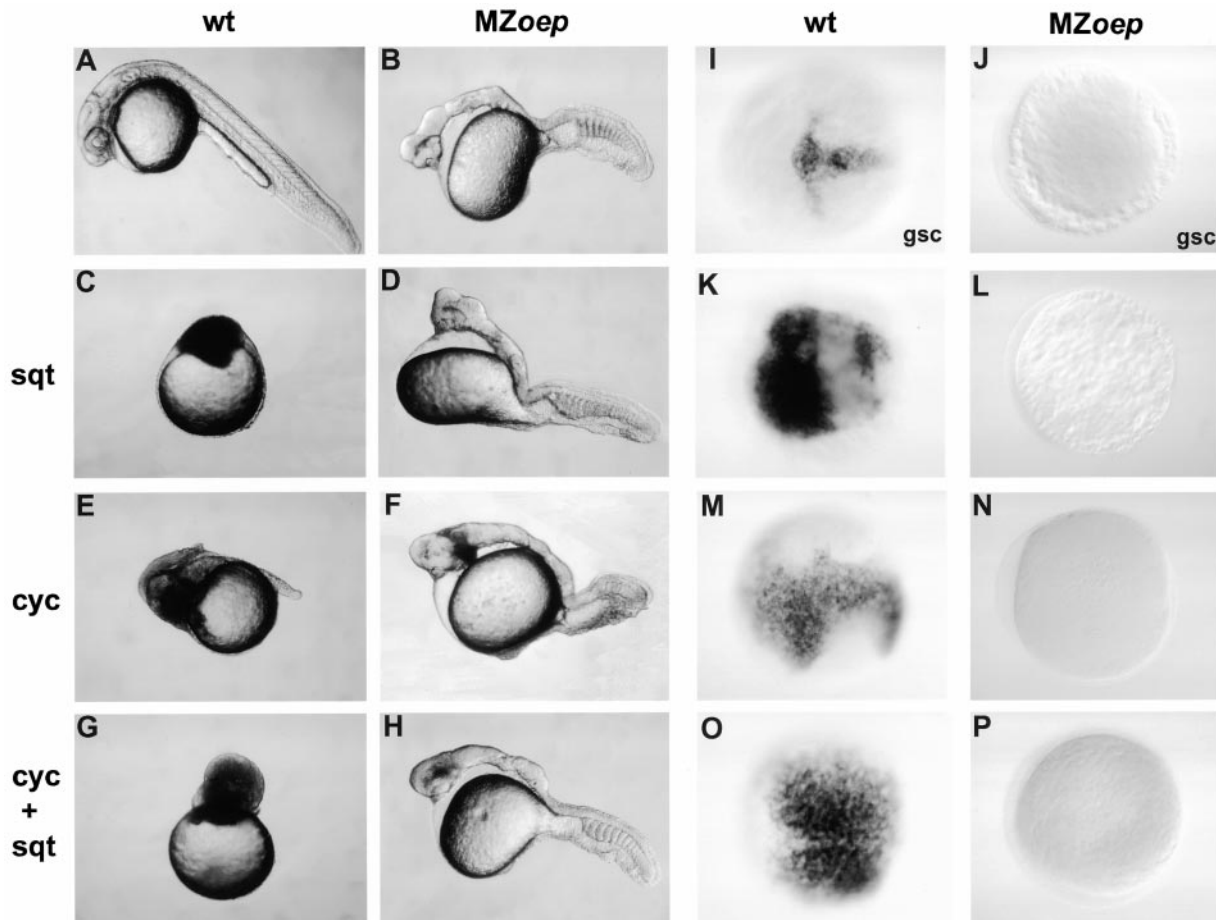


Figure 4. Dependence of Nodal Signaling on Oep Activity

(A–H) Live embryos at 30 hpf, anterior to the left, dorsal up. (I–P) *gsc* mRNA expression in embryos at 90% epiboly, animal pole view. (A and I) Uninjected wild type; (B and J) Uninjected MZOep mutant embryo. (C, E, G, K, M, and O) Wild-type embryos and (D, F, H, L, N, and P) MZOep mutant embryos from a homozygous *oep*^{ts7} intercross, microinjected at the 1–4 cell stage with 10 pg of *sqt* mRNA (C, D, K, and L), 10 pg of *cyc* mRNA (E, F, M, and N), 25 pg of *cyc* and 25 pg of *sqt* mRNA (G and H), or 10 pg of *cyc* and 10 pg of *sqt* mRNA (O and P). Note the severe dorsalization and ectopic *gsc* expression in wild-type embryos injected with *sqt* (C and K), *cyc* (E and M), or *cyc* and *sqt* (G and O) mRNA. In contrast, MZOep embryos injected with *sqt* (D and L) or *cyc* (F and N) mRNA are indistinguishable from uninjected siblings (B).

maternal Activin in MZOep mutants are too low to induce mesoderm formation in the absence of Nodal signaling.

The EGF-CFC Genes *cripto* and *FRL1* Rescue *oep* Mutants

The results presented above indicate that Oep is an essential cofactor for Nodal signaling. Oep and other EGF-CFC proteins share only ~30% sequence identity and have been implicated in seemingly unrelated functional contexts (reviewed in Salomon et al., 1999). We thus hypothesized that EGF-CFC proteins might have different *in vivo* activities. To test this idea, we injected mRNA encoding mouse Cripto and frog FRL1 into MZOep mutants. Despite the divergent sequence of Cripto and FRL1, MZOep mutants are rescued and no dominant phenotypes are induced upon overexpression of these factors (Figure 1E and data not shown). These results establish that mouse Cripto, frog FRL1, and zebrafish Oep can elicit the same biological responses *in vivo*.

Oep Acts Extracellularly between Signaling and Responding Cells

Previous *in vitro* studies have suggested that soluble Cripto protein acts as a nonautonomous signal (Salomon et al., 1999). In contrast, Oep is membrane attached (Zhang et al., 1998) and acts cell autonomously in prechordal plate (Schier et al., 1997) and floor plate development (Strähle et al., 1997), derivatives of mesoderm and ectoderm, respectively. To address the possibility that this apparent discrepancy reflects cell type-specific action of EGF-CFC proteins, we first tested the autonomy of Oep function in endoderm development. Analysis of genetic mosaics shows that wild-type donor cells form *axial*-expressing endoderm progenitors in *oep* mutant hosts, whereas *oep* mutant donor cells cannot develop into endoderm in wild-type hosts (Figures 6A–6C and data not shown). These results indicate that endogenous, membrane-attached Oep acts autonomously in the formation of multiple cell types, including prechordal plate, floor plate, and endoderm.

Table 1. Role of Oep during Nodal Signaling

Genotype	RNA	Amount (pg)	No. Injected	No. Dorsalized at 30 hpf	No. Rescued at 30 hpf	Goosecoid Expressed	Goosecoid Expanded
wt	<i>ActRIB</i>	1	40	0/40	NA	ND	ND
MZ <i>oep</i>		1	33	0/18	0/18	0/15	0/15
wt	<i>ActRIB*</i>	4	12	12/12	NA	ND	ND
MZ <i>oep</i>		4	23	23/23	23/23	ND	ND
wt		2	19	9/9	NA	10/10	10/10
MZ <i>oep</i>		2	54	26/26	26/26	25/28	25/28
wt		1	25	10/12	NA	13/13	13/13
MZ <i>oep</i>		1	33	2/18	15/18	10/15	10/15
wt		0.1	28	8/14	NA	14/14	13/14
MZ <i>oep</i>		0.1	14	0/14	7/14	ND	ND
wt	<i>smad2*</i>	100	28	28/28	NA	ND	ND
MZ <i>oep</i>		100	16	16/16	16/16	ND	ND
wt		50	45	22/22	NA	23/23	21/23
MZ <i>oep</i>		50	42	16/16	16/16	26/26	26/26
wt		10	26	16/18	NA	8/8	7/8
MZ <i>oep</i>		10	40	9/19	18/19	21/21	21/21
wt		4	21	6/11	NA	10/10	9/10
MZ <i>oep</i>		4	31	4/21	14/21	10/10	10/10
wt		1	23	8/12	NA	11/11	11/11
MZ <i>oep</i>		1	30	5/21	19/21	8/9	8/9
wt	<i>activin</i>	2.5	70	14/24	NA	46/46	40/46
MZ <i>oep</i>		2.5	70	37/37	37/37	30/33	30/33
MZ <i>oep</i>		0.5	26	0/11	9/11	3/15	0/15
MZ <i>oep</i>		0.1	34	0/19	1/19	0/15	0/15
wt	mouse <i>nodal</i>	50	27	25/27	NA	ND	ND
MZ <i>oep</i>		50	9	0/9	0/9	ND	ND
wt	<i>sqt</i>	100	24	24/24	NA	ND	ND
MZ <i>oep</i>		100	27	0/27	0/27	ND	ND
wt		10	18	18/18	NA	ND	ND
MZ <i>oep</i>		10	42	0/42	0/42	ND	ND
wt		10	61	28/29	NA	32/32	32/32
MZ <i>oep</i>		10	42	0/27	0/27	0/15	0/15
wt		0.1	46	14/46	NA	ND	ND
wt		0.1	19	ND	NA	19/19	1/19
wt		0.05	21	ND	NA	21/21	3/21
wt	<i>cyc</i>	10	60	41/41	NA	19/19	18/19
MZ <i>oep</i>		10	34	0/22	0/22	0/12	0/12
wt	<i>cyc+sqt</i>	25 + 25	44	44/44	NA	ND	ND
MZ <i>oep</i>		25 + 25	56	0/56	0/56	ND	ND
wt		10 + 10	40	26/26	NA	14/14	13/14
MZ <i>oep</i>		10 + 10	29	0/15	0/15	0/14	0/14
wt		0.1 + 0.1	41	35/41	NA	ND	ND

Phenotypes upon expression of *ActRIB*, activated *ActRIB* (*ActRIB**), activated *smad2* (*smad2**), *activin*, *nodal*, *squint*, and *cyclops* in wild-type and MZ*oep* mutant embryos. Each row represents one experiment, in which embryos injected with the same batch of mRNA were either fixed at 90% epiboly for in situ hybridization with a *gsc* probe or were observed under a dissecting microscope at 30 hpf. Dorsalized embryos at 30 hpf had any of the following characteristics: enlarged hatching gland, enlarged notochord, short twisted tail, extra anterior neural structures, or a partial or complete secondary axis. Severely dorsalized embryos had no discernible axis and were usually dead at 30 hpf. Rescued MZ*oep* embryos at 30 hpf had any of the following structures: two eyes, ventral forebrain, hatching gland, notochord, or trunk somites. For wild-type injections, crosses between two wild-type parents were used. Note that wild-type embryos are more sensitive to lower doses of the injected reagents. This might be due to the endogenous Nodal signaling already present in these embryos. For MZ*oep* injections, crosses between two *oep/oep* parents were used, yielding 100% MZ*oep* mutant embryos. For *nodal*, *sqt*, *cyc*, and *cyc+sqt* injections into MZ*oep* embryos, wild-type controls were always injected with the same amount of the same batch of mRNA on the same day. ND, not determined; NA, not applicable, that is, wild-type embryos cannot be rescued.

To account for the autonomy of membrane-attached Oep and the nonautonomous role of soluble Cripto derivatives, we hypothesized that EGF-CFC proteins act solely extracellularly to mediate signaling between cells. In this scenario, the autonomy of Oep is caused by its membrane attachment, whereas soluble Cripto derivatives could act nonautonomously in vitro. This hypothesis predicts that a secreted form of Oep could function

nonautonomously. To test this idea, we injected mRNA encoding a secreted, C-terminally truncated form of Oep (Oep^{m134}; Zhang et al., 1998) into the YSL (Figure 6E). Secreted protein is thus synthesized extraembryonically outside of cells that respond to Nodal signals. As shown in Figure 6F, secreted Oep nonautonomously rescued MZ*oep* mutants, allowing for the formation of two eyes and a hatching gland. This phenotype was not observed

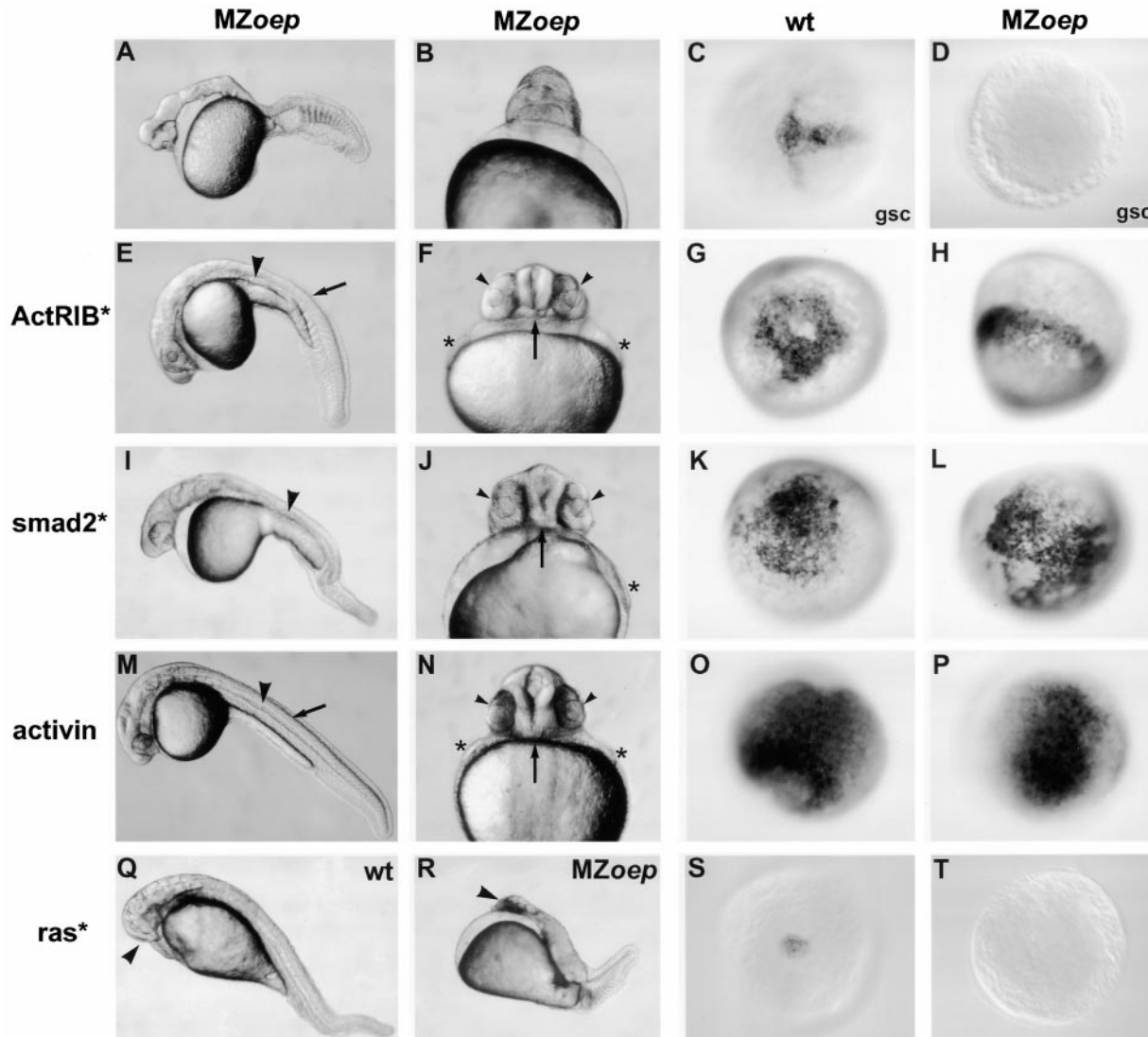


Figure 5. Suppression of the Maternal-Zygotic *oep* Mutant Phenotype by Activation of the the Smad2 Signaling Pathway

Live MZoep (A, B, E, F, I, J, M, N, and R) and wild-type (Q) embryos at 30 hpf. (A, E, I, M, Q, and R) Lateral views, with anterior to the left, dorsal up. (B, F, J, and N) Ventral views of the head. (C, D, G, H, K, L, O, P, S, and T) Animal pole views of embryos at 90% epiboly following whole-mount in situ hybridization with a *gsc* probe. (C, G, K, O, and S) Wild-type embryos. (D, H, L, P, and T) MZoep mutant embryos. (A–D) Uninjected siblings. Phenotype after injection of 1 pg activated *ActRIB* (*ActRIB**; [E and F]) or activated *smad2* (*smad2**; [I and J]) mRNA, 0.5 pg of *activin* (M and N) mRNA, or 1 pg of dominant activated *ras* (*ras**; [Q and R]) mRNA. Note rescue, such as two eyes (arrowheads in [F], [J], and [N]), ventral forebrain (arrow in [F], [J], and [N]), hatching gland (asterisk in [F], [J], and [N]), notochord (arrowhead in [E], [I], and [M]), and trunk somites (arrow in [E] and [M]). Expression of activated *ras* leads to reduction of anterior development in wild-type ([Q], 19/21 embryos) and MZoep mutants ([R], 33/38). Arrowhead highlights absence of eyes (Q and R). *gsc* expression after injection of 2 pg *ActRIB** mRNA (G and H), 1 pg of *smad2** mRNA (K and L), and 2.5 pg *activin*-B mRNA (O and P). Injection of 10 pg of dominant activated *ras* represses *gsc* expression in wild type ([S]; 38/52 injected embryos) and does not rescue expression in MZoep mutant ([T]; *gsc* expression is detectable in 0/47 injected embryos). Activated *ActRIB* corresponds to human *ActRIB* (ALK4) harboring a T206E mutation in the GS domain; activated *Smad2* corresponds to a fusion of β -galactosidase with N-terminally truncated mouse *Smad2*; *Activin*-B is from *Xenopus*. Dominant activated *Ras* is p21^{V-Ha-Ras} from Whitman and Melton (1992).

upon expression of full-length *Oep* or *Cripto* in the YSL (data not shown). These results support the idea that EGF-CFC proteins act solely as extracellular factors.

Discussion

Oep Is an Essential Cofactor for Nodal Signals

We report here the identification of *oep* as a maternal-zygotic effect gene required for vertebrate embryogenesis. Our studies establish that *Oep* acts as an essential

extracellular factor during germ layer formation, organizer development, and the positioning of the anterior-posterior axis. Four lines of evidence identify *Oep* as a novel and specific component of the Nodal signaling pathway. First, the phenotype of MZoep mutants is very similar or identical to the phenotype of double mutants for the *nodal*-related genes *sqt* and *cyc*. Secondly, MZoep mutants do not respond to the overexpression and misexpression of Nodal signals. Third, activation of the putative Nodal signaling pathway by introduction of

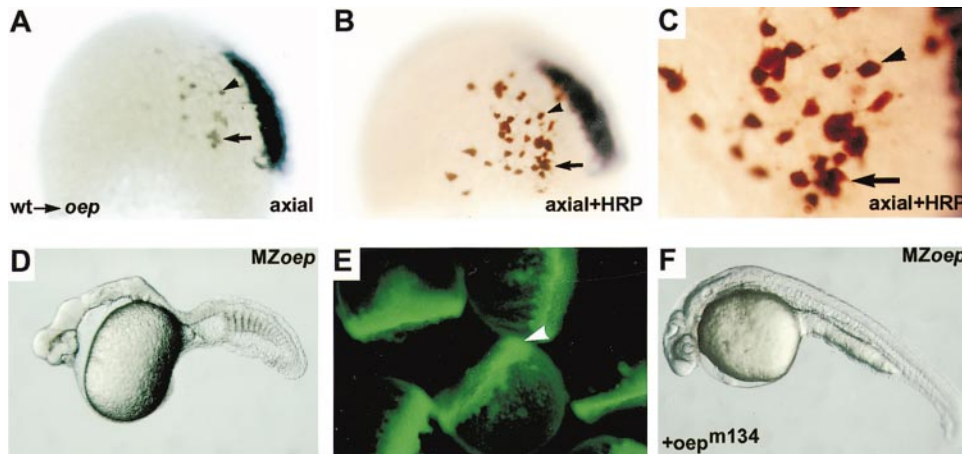


Figure 6. Autonomous Role of Endogenous Oep and Nonautonomous Activity of Secreted Oep

(A–C) Oep acts cell autonomously in endoderm progenitors. Side views of the same *Zoep* host harboring wild-type donor cells, with dorsal to the right, expressing *axial* in the axial mesoderm. Genetic mosaics were generated by transplanting at high stage a small number of wild-type cells (<20) labeled with fluorescein-dextran into unlabeled hosts from an *oep*^{ts7} heterozygous intercross. Hosts were fixed at 80% epiboly and subjected to whole-mount in situ hybridization with the *axial* probe to visualize endoderm precursors (Schier et al., 1997). Homozygous *Zoep* hosts were identified by the lack of widespread *axial* expression. (A) *Zoep* mutant after in situ hybridization. Note the cells that are not located in the midline but express *axial* (arrow and arrowhead). Forty out of seventy *oep* mutant hosts with wild-type donor cells contained *axial*-expressing cells outside the midline domain. To visualize the donor cells, hosts were then stained with an anti-fluorescein HRP antibody. (B) The wild-type donor cells appear brown following HRP staining. (C) High-magnification view of the embryo in (B). Note that *axial*-expressing endoderm precursors (arrow and arrowhead) are from donor embryos, as judged from brown HRP staining. *axial*-expressing endoderm precursors corresponded to donor cells in 40 out of 40 *Zoep* mutant hosts. In the reverse transplant, *Zoep* mutant donor cells in wild-type hosts never became specified as endoderm precursors (0/29; data not shown). In wild-type into wild-type transplants, 15/33 host embryos harbored *axial*-expressing donor-derived endoderm precursors.

(D–F) Localized expression of a secreted, C-terminally truncated form of Oep (Oep^{m134}; Zhang et al., 1998) in the yolk syncytial layer (YSL) can rescue the MZoop phenotype. (D and F) MZoop embryos at 30 hpf, anterior to the left. (D) Uninjected MZoop mutant. (E) The lineage tracer fluorescein-dextran is only seen in the YSL following injection (white arrowhead) and in the yolk at 30 hpf (not shown). (F) MZoop mutant after YSL injection of *oep*^{m134} mRNA. Note the rescue of MZoop mutants (rescue in 74 out of 74 injected embryos). Embryos from an *oep*^{ts7} homozygous intercross were coinjected into the YSL with 50 pg of *oep*^{m134} mRNA and 25 pg fluorescein-dextran at the high stage.

activated forms of ActRIB and Smad2 or by expression of Activin can rescue various aspects of the MZoop mutant phenotype. Fourth, the *oep* mutant phenotype is distinct from those induced by blocking or ectopically activating the BMP2/4, wnt/ β -catenin, or Ras/MAPK pathways (reviewed in Harland and Gerhart, 1997; Schier and Talbot, 1998), suggesting that Oep is primarily involved in signaling by Nodal-like factors. This conclusion is further supported by the finding that activation of the Ras/MAPK, BMP2/4, and wnt/ β -catenin signaling pathways does not rescue the *oep* mutant phenotype (Figure 5; Schier et al., 1997; D. Lee, W. S. T., and A. F. S., unpublished results).

How does Oep function as an essential component of Nodal signaling? Two principal mechanisms can be envisioned. Oep either acts as a positive cofactor that allows Nodal to activate its downstream signaling pathway or as a negative factor that counteracts an inhibitor of Nodal signaling. The latter scenario would be comparable to the role of Tolloid and Xolloid, which protect Dpp/BMP4/BMP2 from sequestration by short gastrulation/chordin (Blader et al., 1997; Marqués et al., 1997; Piccolo et al., 1997). In contrast to Tolloid, however, overexpression of Oep does not induce obvious dominant phenotypes (Zhang et al., 1998). In particular, we do not detect severe dorsalization or expansion of notochord or prechordal plate as seen upon augmented Nodal signaling. Secondly, the defects caused by absence of Tolloid can be suppressed by overexpression of Dpp (Ferguson and Anderson, 1992). In contrast,

MZoop mutants are unresponsive to Nodal overexpression.

Our results are more compatible with a role for Oep as a cofactor for Nodal signals, similar to the role of the GFR α 1 protein. GFR α 1 serves as an extracellular coreceptor for the glial cell line-derived neurotrophic factor (GDNF; Jing et al., 1996; Treanor et al., 1996). The interaction of GFR α 1 with GDNF, a signaling molecule with a TGF β fold, mediates binding and activation of the Ret tyrosine kinase receptor. Both Oep and GFR α 1 are membrane attached, permissive, and required extracellularly for the activity of an instructive signal. Based on these parallels, we propose that Oep acts as an essential extracellular cofactor for Nodal signals, much as GFR α 1 mediates GDNF signaling.

Nodal and Oep Activate an Activin-like Signaling Pathway

Genetic studies in mouse and misexpression studies in frog have led to the proposal that Nodal signaling is mediated via the Activin receptors ActRII/ActRIIB and ActRIB and the transcription factor Smad2 (Oh and Li, 1997; Gu et al., 1998; Nomura and Li, 1998; Weinstein et al., 1998; reviewed in Harland and Gerhart, 1997; Whitman, 1998). Other reports, however, have suggested that different or additional members of the TGF β family activate the Activin receptors and Smad2 in vivo (e.g., Dyson and Gurdon, 1997; Waldrup et al., 1998). Since we have not detected a stable physical interaction between Nodal, Oep, and the type II and I Activin receptors in

vitro (J. Z., W. S. T., and A. F. S., unpublished results), the detailed molecular interactions during Nodal signaling remain to be elucidated. Nevertheless, our finding that activation of ActRIB and Smad2 rescues the defects in Nodal signaling of *MZoep* mutants strongly supports the idea that these or similar TGF β pathway components are involved in transmission of Nodal signals. Moreover, we find that ectopic expression of Activin bypasses the dependence of Nodal signaling on Oep activity and rescues *MZoep* mutants. Previous in vitro studies have shown that Activin directly activates ActRIB, ActRII, ActRIIB, and Smad2 (reviewed in Harland and Gerhart, 1997; Massagué, 1998; Whitman, 1998). These results suggest that Activin mimics the combined activity of Nodal and Oep in activating an Activin-like pathway.

Multiple Roles for Oep-Mediated Nodal Signaling during Development

Two lines of evidence suggest that Nodal signals are dependent on Oep throughout zebrafish development. First, *oep* and *sqt/cyc* are consistently expressed in overlapping domains, including the regions of the blastula margin, axial mesoderm, ventral neuroectoderm, forebrain, lateral plate mesoderm, and epithalamus (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998b; Sampath et al., 1998; Zhang et al., 1998). Second, genetic studies have shown that *oep* is required in patterning events thought to involve Nodal signals, including germ layer formation, floor plate development, ventral forebrain formation, and left-right patterning (Hatta et al., 1991; Heisenberg and Nüsslein-Volhard, 1997; Schier et al., 1997; Strähle et al., 1997; Varlet et al., 1997; Feldman et al., 1998; K. G., W. S. T., and A. F. S., unpublished results). These results suggest a general dependence of Nodal signals on Oep throughout zebrafish embryogenesis. We propose that Oep serves as a competence factor that allows Oep-expressing cells to respond to Nodal, while nonexpressing cells are protected from Nodal signaling.

Direct Role for Nodal Signaling in Floor Plate Induction

The finding that Oep is required for signaling by Cyc resolves a controversy surrounding the role of Nodal signaling in floor plate induction. No ventral neuroectoderm, including floor plate, forms in zygotic *oep* and *cyc* mutants. Partial rescue of floor plate development in *cyc* mutant embryos can be achieved by expression of *cyc* mRNA in non-floor plate cells, suggesting that Cyc acts nonautonomously during floor plate induction (Sampath et al., 1998). It has been unclear, however, if Cyc acts as a direct inducer of floor plate in zebrafish. For example, one possible alternative is that Cyc allows normal formation of axial mesoderm that subsequently induces floor plate cells in a Nodal-independent mechanism (Dodd et al., 1998). Previous studies have indicated that Oep is required cell autonomously in floor plate precursors (Strähle et al., 1997). Together with our finding that Oep acts as an essential cofactor for Cyc signaling, these results provide strong support for a direct role of Nodal signaling in zebrafish floor plate induction.

Common In Vivo Specificity of EGF-CFC Proteins

We have found that injection of mouse *cripto* and frog *FRL1* mRNA rescues *MZoep* mutants, rendering them responsive to Nodal signals. Thus, EGF-CFC genes appear to encode a family of proteins that have common in vivo specificities and serve as general cofactors for Nodal-like signals. These results are surprising in light of previous reports that suggested that Cripto and FRL1 constitute EGF-like signals that lead to Ras/Raf/MEK/MAPK activation (reviewed in Salomon et al., 1999). While we find no evidence that Oep is required for Ras in vivo activity, the proposed signaling function of Cripto can be reconciled by the fact that a locally secreted, mutant form of Oep (Oep^{m134}) can function nonautonomously, that is, similar to a signal. This finding reflects the extracellular function of Oep as a component of Nodal signaling between Nodal-secreting and -responding cells. Secretion of Oep is artificial, however, as wild-type Oep is membrane attached (Zhang et al., 1998) and acts cell autonomously in prechordal plate (Schier et al., 1997), floor plate (Strähle et al., 1997), and endoderm formation (Figure 6). These results suggest that Cripto does not act as an instructive signal in MAPK signaling, but that addition of soluble Cripto protein renders cells competent to respond to an instructive signal such as Nodal or another TGF β ligand.

While maternal-zygotic *oep* mutants and *sqt/cyc* double mutants in zebrafish display identical phenotypes, the similarity between mouse *cripto* and *nodal* mutants appears to be less obvious (Beddington and Robertson, 1999). For instance, *nodal*, but not *cripto*, mutants appear to lack the anterior neuroectoderm. It is interesting to note, however, that both mutants have common defects in primitive streak formation and mesoderm and endoderm development. The stronger phenotype of mouse *nodal* mutants might be explained by the presence of other EGF-CFC genes, such as mouse *cryptic* (Shen et al., 1997), which could allow partial Nodal signaling in *cripto* mutants. In addition, it is possible that a more detailed analysis of *nodal* (and *ActRIB* and *smad2*) mutants may uncover further similarities with *cripto* mutants.

Conserved Function of EGF-CFC Genes in Vertebrate Germ Layer Formation and Positioning of the Anterior-Posterior Axis

Comparison of the zebrafish *MZoep* and mouse *cripto* mutant phenotypes reveals surprising similarities. In contrast to *cripto* mutants, *MZoep* mutants develop a tail and hindbrain, but both mutants display defective germ layer and trunk formation. Furthermore, the position of the anterior-posterior axis appears to be abnormal in both *MZoep* and *cripto* mutants. Both mutant phenotypes can be interpreted as an $\sim 90^\circ$ displacement of the anterior-posterior embryonic axis in relation to its normal position (compare Figure 2 in this report with Figure 4 in Ding et al., 1998). It has been proposed that *cripto* mutations interfere with pregastrulation cell movements in the visceral (extraembryonic) endoderm, which then leads to the abnormal induction of the brain at a distal instead of anterior position (Ding et al., 1998; reviewed in Beddington and Robertson, 1999). In contrast, our studies in *MZoep* mutants indicate that abnormal axis positioning is preceded by defects in embryonic

patterning at blastula stages. Time course analysis suggests that ventral and lateral marginal cells fail to form involuting endoderm and trunk mesoderm and instead solely contribute to the tail in *MZoep* mutants. Similarly, the abnormal location of the hindbrain might be caused by an early specification defect wherein dorsal marginal cells, which normally give rise to axial mesoderm, acquire a hindbrain fate. These observations suggest that abnormal specification of blastoderm marginal cells might be the primary cause of abnormal axis positioning in *MZoep* mutants. Despite potential differences in morphogenetic movements, the phenotypes of *cripto* and maternal-zygotic *oep* mutants suggest that vertebrate germ layer formation and positioning of the anterior-posterior axis is controlled by conserved molecular mechanisms involving EGF-CFC proteins.

Experimental Procedures

Strains

Adult fish homozygous for *oep*^{z57} (Hammerschmidt et al., 1996), a null allele (Zhang et al., 1998), were initially obtained by microinjection of mRNA encoding *Oep* into embryos from a cross of *oep*^{z57/+} parents (Zhang et al., 1998). Crossing to *oep*^{z57/+} fish then identified homozygous adults. Adult *oep/oep* fish were later obtained by microinjection of *oep* mRNA into embryos from a homozygous intercross, so that all surviving fish were homozygous. Of 129 *MZoep* mutant embryos that appeared morphologically wild type at 72 hr postfertilization (hpf) upon injection of *oep* mRNA, 98 survived to adulthood. Embryos were staged according to Kimmel et al. (1995).

Phenotypic Analysis

For time course analysis, embryos were mounted at shield stage and oriented in a lateral view in 3.5% methylcellulose (wild type) or 0.1% agarose/0.02% agar (*MZoep*). Positions of animal and vegetal poles were labeled on the coverslip with a fine tip marker, and embryos were photographed at regular intervals until the 20S stage (S, somite) in the same position using a Leica MZAP0 dissecting microscope. In situ hybridization was performed as described (Zhang et al., 1998). For references to RNA probes, see Schier et al. (1997) and Feldman et al. (1998).

RNA Microinjections

The following plasmids were linearized, and sense strand-capped mRNA was synthesized using the mMESSAGE mMACHINE system (Ambion): pSP64T-XALK4 (*Xenopus* ActRIB; digested with EcoRI, transcribed with SP6; Chang et al., 1997), pSP64T-X β nodal (mouse *nodal*; EcoRI, SP6; Toyama et al., 1995), pSP64T-Xactivin β -B (*Xenopus* activin β -B; EcoRI, SP6; Sokol et al., 1991), pSP64T-ALK4-HA-T206E (activated human ActRIB; XbaI, SP6; Chang et al., 1997), pCS+cyt β gal-madr2(C) (β -galactosidase fusion with N-terminally truncated mouse *Smad2*; XbaI, SP6; Baker and Harland, 1996), pCS2+cyclops (NotI, SP6; Rebagliati et al., 1998a), pCS2+squnt (NotI, SP6; Feldman et al., 1998), pCDNA3oep (NotI, T7; Zhang et al., 1998), and pCDNA3oep^{m134} (C-terminally truncated *Oep*; Smal, T7; Zhang et al., 1998). pT7TS-Cripto and pT7TS-FRL1 (Cripto and FRL1 coding regions, respectively, in Spe1 site of pT7-TS; BamHI, T7); pSP64T-Ha-v-Ras (dominant active Ras; EcoRI, SP6; Whitman and Melton, 1992). RNA was diluted in 5 mg/ml phenol red in 0.2 M KCl and microinjected into the yolk or blastomeres at 1–4 cell stage or into the YSL at high stage of embryos that had been dechorionated by pronase treatment. The volume of injected RNA was determined by measuring the diameter of a spherical drop of RNA injected into mineral oil on an objective micrometer with 0.01 mm divisions (WARDS). Genotyping of injected embryos was not necessary, as all embryos obtained from an *oep*^{z57} homozygous intercross have the genotype *oep/oep*.

Cell Transplantation

Genetically mosaic embryos were generated using cell transplantation techniques, as described previously (Schier et al., 1997), with

some modifications. Donor embryos were labeled with 5% fluorescein-dextran (Molecular Probes). Following transplantation, host embryos were fixed at 80% epiboly and processed by in situ hybridization with the *axial* riboprobe, using NBT-BCIP as a substrate. After in situ hybridization, embryos were photographed in 3.5% methylcellulose in PBT with a Leica MZAP0 dissecting microscope. After several washes in PBT, donor cells were detected in the same embryos by immunostaining with the anti-fluorescein-HRP antibody (Boehringer Mannheim), using DAB as a substrate. Embryos were then cleared in benzyl benzoate/benzyl alcohol, mounted in Permount, and photographed on a Zeiss Axiophot microscope.

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